



The Antibacterial Activity of Honey and Lemon Juice against *Streptococcus pneumoniae* and *Streptococcus pyogenes* Isolates from Respiratory Tract Infections

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Abstract

This study was aimed to determine the antibacterial activity of honey and/or lemon juice on strains of *Streptococcus pneumoniae* and *Streptococcus pyogenes* from respiratory tract infections. Clinical isolates were collected from Ahmadu Bello University Teaching Hospital (ABUTH), Zaria and Ahmadu Bello University Health Services (ABUHS) Samaru campus, Zaria. The isolates were characterized by standard microbiological procedures. Antibacterial activity of the honey and lemon juice, as well as that of some standard antibiotic formulations were assayed using agar well diffusion and broth dilution method. Minimum Inhibitory and Bactericidal Concentrations were carried out. Rate of kill was also carried out to determine the death/survival rate of the bacterial isolates after exposure to the agents. Noticeable variations in the antibacterial activity of the agents were observed. Thus, inhibition zones (mm) ranging from 10 - 22 (100% Honey), 14 - 29 (100% Lemon) and 20 - 29 (Honey/Lemon juice mixture) were obtained. However, Minimum Inhibitory Concentrations ($\mu\text{g/ml}$) range between 1.95-125 (Ceftriaxone), 1.56-NI (Gentamicin), 31.5-NI (Amoxicillin-Clavulanic acid), 0.98-62.5 (Levofloxacin), 50.0-NI (Azithromycin), 20.0 - 75.0 (100% v/v Honey), 22.5 - 47.5 (100% v/v Lemon juice) and 17.5 - 25.0 (Honey/Lemon juice mixture). However, for the rate of kill, Honey/Lemon juice mixture, Lemon juice effected complete killing at 120 minutes; While, Ceftriaxone, Levofloxacin and Honey produced complete killing at 1440 minutes. Therefore, from the findings, honey/lemon juice mixture, Lemon juice, Levofloxacin, Ceftriaxone and Gentamicin had higher antibacterial activity than Azithromycin, Amoxicillin-Clavulanic acid and Honey. However, for the statistical analysis, at $p \geq 0.05$, there is significant difference between honey/lemon juice mixture and honey. In conclusion, the bacterial isolates were more susceptible to honey/lemon juice mixture, lemon juice, Levofloxacin, Ceftriaxone and Gentamicin; but less susceptible to Azithromycin, Amoxicillin-Clavulanic acid and Honey. Excellent bactericidal activity was observed with honey/lemon juice mixture, lemon juice compared to the honey alone. The findings in this research therefore provides scientific basis to the use of honey and lemon juice as an alternative medicine by the populace in the treatment of respiratory tract infections.

Keywords: Honey; Lemon Juice; Standard Antibiotics; Antibacterial Activity; Bactericidal; Susceptibility

Introduction

Honey is a sweet food made by bees using nectar from flowers. The variety produced by honey bees (the genus *Apis*) is the one most commonly referred to and is the type of honey collected by beekeepers and consumed by humans. The various species include; *Apis andreniformis*, *Apis florea*, *Apis dorsata*, *Apis cerana*, *Apis koschevnikovi*, *Apis mellifera*, *Apis nigrocincta*. Other species include; Stingless bees, sometimes called stingless honey bees or simply meliponines, are a large group of bees (approximately 500 species), comprising the tribe Meliponini or subtribe Meliponina according to other authors [1].

Most microorganisms do not grow in honey because of its low water activity of 0.6 [2]. Hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin, pH, osmotic effect as well as leptosin were known to be responsible for the antimicrobial effects of honey [3,4].

Lemon fruit is an inexpensive, easily available citrus fruit, popular for its culinary and medicinal uses. The Lemon fruit juice consists of about 5% citric acid that gives a sour (tarty) taste to the

lemon [5]. Lemon is an important medicinal plant of the family Rutaceae. It is a rich source of vitamin C and it is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (viz., leaves, stem, root and flower) of Lemon against clinically significant bacterial strains has been reported [6].

Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities [7].

There are different varieties of lemon. This include: Bush lemon tree, Eureka, Lisbon, Meyer, Ponderosa, and Variegated pink. However, the species used in this work was the Eureka species as identified in the Herbarium section of Biological Science, Ahmadu Bello University, Zaria.

Eureka fruit has a markedly ribbed surface. The fruit color is yellow at maturity. It is a sour lemon variety and usually has fewer seeds [5]. The pulp of a Eureka lemon is greenish-yellow.



Plate I: Eureka Lemon Fruits (Moon Growers, 2010).

The respiratory tract is the part of the anatomy involved with the process of respiration [8]. The respiratory tract is divided into the upper and the lower respiratory tract. The upper respiratory tract is generally considered to be the airway above the glottis or vocal cords. This includes the nose, sinuses, pharynx, and larynx [9]. Whereas, the lower respiratory tract consists of the trachea (wind pipe), bronchial tubes, the bronchioles, and the lungs [10]. Infections involving this tract are referred to as respiratory tract infections [11]. Therefore, infection is the commonest and most serious complication of respiratory tract [8]. This includes pharyngitis, sometimes involving tonsillitis, and giving rise to a “sore throat”, nasopharyngitis, otitis media, sinusitis, epiglottitis, bronchitis, pneumonia, etc. These results in significant morbidity, which may account for missed days of work and school, it also contribute to mortality. However, the fact that antimicrobials are being misused for treatment of cold, lemon and honey are considered natural soothers which have been utilized in some of these mild illnesses.

Significance and Health Implication of the Test Organisms
Streptococcus pyogenes

Streptococcus pyogenes is the most common bacterial cause of sore throat [12]. A painful, red throat with white patches on your tonsils is characteristic of pharyngitis, otherwise known as strep throat. It is usually accompanied by swollen lymph nodes, fever, and headache. Occasionally it is also accompanied by nausea, vomiting, and abdominal pain [13].

Beta-hemolytic streptococci produce a toxin that forms a clear zone of hemolysis on blood agar, demonstrating its ability to destroy red blood cells [14]. This hemolysis is attributed to toxins formed by Group A streptococci called streptolysins.

It is estimated that there are more than 700 million infections (caused by *Streptococcus pyogenes*) worldwide each year and over 650,000 cases of severe, invasive infections that have a mortality rate of 25% [15]. Early recognition and treatment are critical; diagnostic failure can result in sepsis and death [16].

Diseases caused by *Streptococcus pyogenes* usually respond well to antibiotic treatment. The American Heart Association and the Infectious Diseases Society of America recommend penicillin as the drug of choice for treatment [17].

Streptococcus pneumoniae

Streptococcus pneumoniae, or pneumococcus, is a Gram-positive, alpha-hemolytic, aerotolerant anaerobic member of the genus *Streptococcus* [18].

Despite the name, the organism causes many types of pneumococcal infections other than pneumonia. These invasive pneumococcal diseases include acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess [19]. It is also one of the top two isolates found in ear infection, otitis media [20].

Worldwide in 2000, 14.5 million estimated episodes of invasive pneumococcal disease were reported in children younger than 5 years of age, which correlates to an estimated more than 800,000 deaths (11% of all deaths in this age group) [18].

Materials and Methods

Methodology

Study area

The study areas were Ahmadu Bello University Teaching Hospital (ABUTH) and Ahmadu Bello University Health Services (ABUHS) Zaria, Kaduna State, Nigeria. However, the research was conducted in the Faculty of Pharmaceutical Sciences, Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University (ABU), Zaria, Nigeria.

Collection of materials and samples

Pure honey was collected from Taraba State, Nigeria. However, the lemon (*Citrus limon*, Eureka variety) was obtained from the Staff quarters in Area-A, ABU, Zaria.

Isolation and identification of bacteria from respiratory tract infections

Clinical isolates from sputum, throat, ear swab and nasal secretions samples were collected in ABUTH, Zaria and ABUHS, Samaru Campus, Zaria. These were then inoculated on Blood Agar, Chocolate agar, MacConkey agar and cetrimide agar, and the plates incubated at 37°C for 24 - 48 hours. Identification of the growing microorganisms was done by colony morphology and Gram-Staining method. Pure colonies were sub-cultured on Blood agar, Nutrient agar and Chocolate agar media. Further identification or confirmation was carried out using Biochemical tests as recommended by [21].

Antibacterial activity testing

The honey was diluted with sterile distilled water to concentrations of between 25% (v/v) to 50% (v/v). The lemon was washed with water to remove sand and other particles and rinsed with sterile distilled water. It was cut with sterile knife before the juice was squeezed out and sieved. The sieving was done to remove the seeds and other particles. The juice was diluted with sterile distilled water to concentrations of between 25% (v/v) to 50% (v/v). However, for the combination studies, ratio of mixtures (Lemon juice:Honey:water) was as follows 10:50:40; 20:50:30; 30:50:20; 40:50:10; 50:50:0 (v/v) concentrations and Honey:Lemon juice:water at 10:50:40; 20:50:30; 30:50:20; 40:50:10; 50:50:0 (v/v) concentrations.

Agar well diffusion technique as described by Adeniyi., *et al.* [22] and Adeshina., *et al.* [23] was used to determine the antibacterial activities of the Honey, Lemon juice and the combinations of the two agents. 20 mls of Mueller Hinton agar were prepared and poured into sterile petri-dishes, and then allowed to set. Overnight culture of the test organism which was diluted in sterile normal saline to match 0.5 McFarland turbidity [24] was then spread thinly with sterile swab stick on the surface of the agar. Thereafter, holes were bored using sterile cork-borer (number 4) to make uniform wells on the inoculated agar. The bottom of the hole was then sealed with 2 drops of molten sterile Mueller Hinton agar and then filled with the test antibacterial agent (honey, lemon juice, honey/lemon juice). The standard antibiotic discs were placed at some points in the same Petri dishes with the test antibacterial agents (Honey and/or Lemon juice) for them to undergo the same conditions. Pre-incubation diffusion time (45 minutes to 1 hour) was allowed, after which the petri-dishes were incubated at 37°C for 18 - 24 hrs. After the incubation period, the diameters of the zones of inhibition were measured in millimetres. Interpretation of zones sizes in terms of sensitivity or susceptibility, and resistance was based on the values provided by Clinical and Laboratory Standard Institute (CLSI) [25].

Isolation and identification of bacteria from respiratory tract infections

Concentration (MBC) of the agents

The MIC was carried out using the broth dilution method as used by Kabir., *et al.* [26] and as described by CLSI [25]. Stock solu-

tions of 125 µg/ml were prepared for CRO, AMC, and LEV; 100 µg/ml and 50 µg/ml was also prepared for CN and AZM respectively based on their different MIC break point values. Two fold serial dilution of the stock solutions were made in eight (8) test tubes (plus three control test tubes; one containing Mueller Hinton broth and the test bacteria, another containing Mueller Hinton broth and the standard antibiotics and the other containing Mueller Hinton broth and Sterile distilled water) of Mueller Hinton broth, with the first test tube being a double strength and the others single strength to obtain concentrations between 125 - 0.98 µg/ml, 100 - 0.78 µg/ml and 50.0 - 0.39 µg/ml. However, for the Honey and Lemon juice, 100 µg/ml, 90 µg/ml, 80 µg/ml, 70 µg/ml and 60 µg/ml of stock solutions were prepared. Two fold serial dilution of the stock solutions were also made (same procedure as above) to obtain concentrations between 100 - 0.78 µg/ml, 90.0 - 0.71 µg/ml, 80.0 - 0.63 µg/ml, 70.0 - 0.55 µg/ml and 60.0 - 0.47 µg/ml. The overnight cultures of the test bacterial isolates were diluted to match 0.5 Mc Farland turbidity. At this point, the organisms should be at a concentrations of approximately 10⁵ cfu/ml. An aliquot of 100 µl of the standardized inoculum was then inoculated to the different dilutions of the agents and the antibiotics in the 8 test tubes plus the organism-control test tube and incubated at 37°C for 24 hours. The lowest concentration (highest dilution) of the honey and/or lemon or the antibiotics which showed clear solution or no visible bacterial growth (i.e. no turbidity) when compared with the control tubes, was regarded as the M.I.C.

However, M.B.C. was determined from the broth dilution tests, by sub-culturing to antibiotic free Mueller Hinton agar (i.e. Mueller Hinton Agar+5% v/v tween 80) from tubes showing no visible growth after 24 hours incubation at 37°C. The lowest concentration of an antibacterial agent that kills more than 99.9% of the initial inoculation after the 24 hours incubation represents the MBC [27].

Rate of kill

Aliquot of 0.1 ml of standardized overnight culture of the test organisms that were susceptible and those that were resistant to the standard antibiotics, honey, lemon juice and mixture of both (approximately 106 cfu/ml) was added to 9.9 ml each of test antibacterial agents (honey and/or lemon juice) formulated with sterile distilled water (using the concentrations of Sub-MIC, Around-MIC, and Above-MIC). This was mixed thoroughly and kept inside Digital shaker bath (Mc Donald Scientific International) at 37°C. At different time interval (0, 30, 60, 120, 240, 360 and 1440 minutes). 1 ml test organism/extract admixture was taken and ten-fold dilution protocol performed with sterile inactivated normal saline (i.e. normal saline with 5% (v/v) Tween 80). These dilutions were then plated out in duplicates on sterile molten Mueller–Hinton agar supplemented with 5% (v/v) Tween 80. The agar plates were then incubated at 37°C for 24 hours. After incubation, colonies observed

were counted with the aid of a Colony Counter (NAPCO Model 630 Portland, Oregon, U. S. A.) [23,28]. These procedures were repeated for Sub-MIC, Around-MIC, and Above-MIC values of levofloxacin and ceftriaxone; as the standard antibacterial agents.

Statistical analysis

The zones of inhibitions obtained from the susceptibility tests carried out were expressed as Mean ± Standard Error of Mean (SEM). The mean zone of inhibition of honey and lemon were also compared with that of the mixture and with that of the various antibiotics using Analysis of Variance (ANOVA) to determine the significant differences. Differences were considered significant if P ≤ 0.05 and not significant if P ≥ 0.05.

Results and Discussion

Results

Sample collection

A total of 126 Clinical isolates were collected from Sputum (83), Throat swab (26), Ear swab (14) and Nasal secretion (3) samples. The isolates identified and confirmed from these samples include 15 *Klebsiella pneumoniae* (26.8%), 14 *Staphylococcus aureus* (25.0%), 2 *Haemophilus influenzae* (3.57%), 12 *Pseudomonas aeruginosa* (21.4%), 7 *Streptococcus pneumonia* (12.5%), 6 *Streptococcus pyogenes* (10.7%). However, for the purpose of this research, more focus will be on *Streptococcus pneumoniae* and *Streptococcus pyogenes*.

Susceptibility pattern of the bacterial isolates to honey and lemon juice

Honey/lemon juice mixture, crude concentrations of honey and lemon juice gave a wider zones of inhibition, while low zone of inhibition was seen with 25% v/v concentration of honey (Figure 1 and 2).

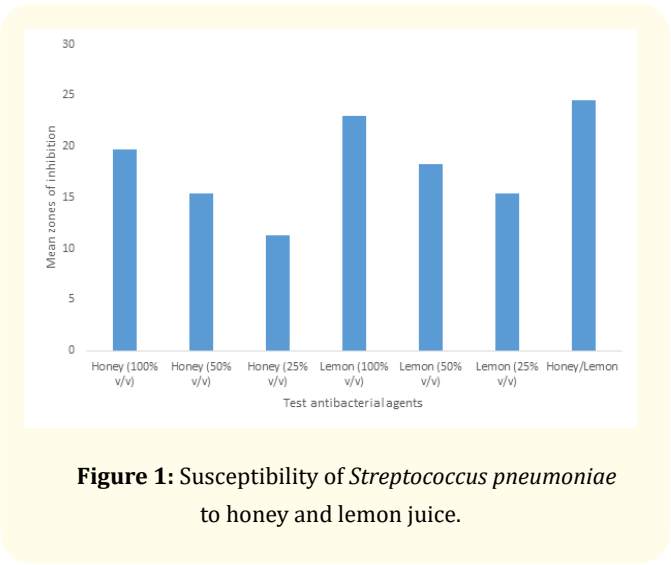


Figure 1: Susceptibility of *Streptococcus pneumoniae* to honey and lemon juice.

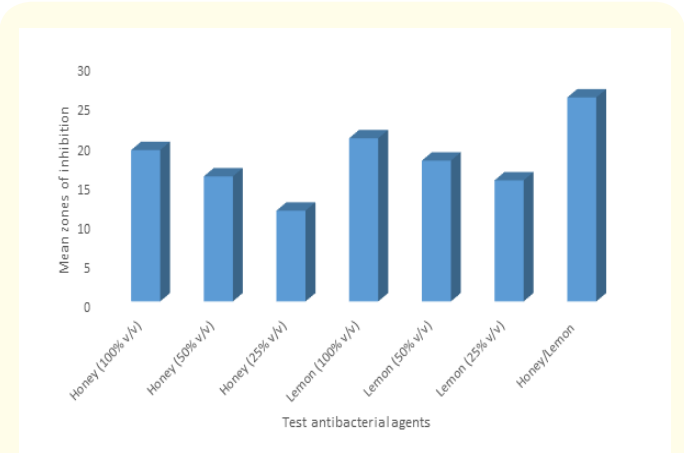


Figure 2: Susceptibility of *Streptococcus pyogenes* to honey and lemon juice.

Comparing the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) results between honey, lemon juice and honey/lemon juice

Generally, there’s reduction in MIC (and MBC) in the mixture of Honey and Lemon juice compared to the Honey alone (Table 1 to 6). There’s also reduction in MIC (and MBC) in the mixture of Honey and Lemon juice compared to the Lemon juice alone in some of the strains while increase in MIC (and MBC) were obtained in the other strains (Table 1 to 8).

Table 1: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Honey and Lemon juice) against *Streptococcus pneumoniae*.

Bacterial isolates	Honey	Lemon
<i>Streptococcus pneumoniae</i>	37.1	31.0

Table 2: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Honey/Lemon juice mixture) against *Streptococcus pneumoniae*.

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
<i>Streptococcus pyogenes</i>	32.9	27.9	25.8	22.5	18.8

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Honey:Lemon juice concentrations (µg/ml).

Table 3: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Lemon juice/Honey mixture) against *Streptococcus pneumoniae*.

Bacterial isolates	Honey	Lemon
<i>Streptococcus pyogenes</i>	43.3	34.6

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Lemon juice:Honey concentrations (µg/ml).

Table 4: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Honey and Lemon juice) against *Streptococcus pyogenes*.

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
<i>Streptococcus pyogenes</i>	20.4	19.6	17.9	17.9	18.8

Table 5: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Honey/Lemon juice mixture) against *Streptococcus pyogenes*

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
<i>Streptococcus pneumoniae</i>	19.6	20.0	20.4	19.6	19.6

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Honey:Lemon juice concentrations (µg/ml).

Table 6: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Lemon juice/Honey mixture) against *Streptococcus pyogenes*

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
<i>Streptococcus pneumoniae</i>	43.6	38.6	27.1	23.9	19.6

Key: 10:50, 20:50, 30:50, 40:50, 50:50=Lemon juice:Honey concentrations (µg/ml).

Table 7: Mean zone of inhibition (mm) ± SEM of the standard Antibiotic formulations against *Streptococcus pneumoniae*.

	CRO	LEV	AMC	AZM	CN
<i>Streptococcus pneumoniae</i>	21.7 ± 1.92	21.3 ± 1.32	19.3 ± 2.07	16.1 ± 1.94	20.4 ± 1.56

Key: CRO: Ceftriaxone; LEV: Levofloxacin; AMC: Amoxicillin-Clavulanic Acid; AZM: Azithromycin; CN: Gentamicin.

Table 8: Mean zone of inhibition (mm) ± SEM of the standard Antibiotic formulations against *Streptococcus pyogenes*

	CRO	LEV	AMC	AZM	CN
<i>Streptococcus pyogenes</i>	20.6 ± 2.27	19.5 ± 1.80	16.3 ± 2.43	15.6 ± 1.42	21.8 ± 0.94

Key: CRO: Ceftriaxone; LEV: Levofloxacin; AMC: Amoxicillin-Clavulanic Acid; AZM: Azithromycin; CN: Gentamicin.

Rate of kill

Honey and Lemon juice mixture, Lemon juice effected complete killing at 120 minutes, as depicted by gradual decrease in the cell population from 30 minutes to 90 minutes and a steady decrease at 120 minutes; while Ceftriaxone, Levofloxacin and Honey produced complete killing at 1440 minutes, as depicted by gradual decrease in cell populations from 30 minutes to 360 minutes and a steady decrease to 1440 minutes for the susceptible *Streptococcus pneumoniae* (Figure 3).

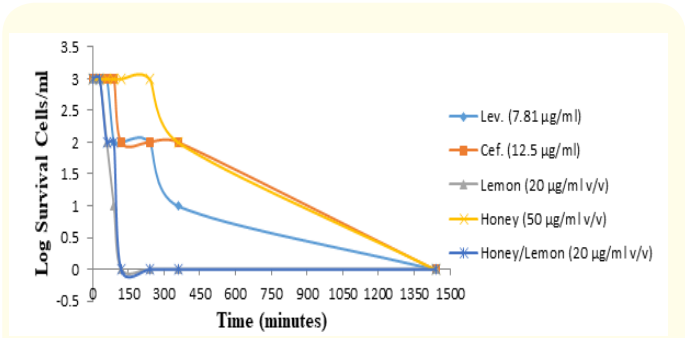


Figure 3: The Log of Survival cells/ml of *Streptococcus pneumoniae* (Sensitive) on exposure to standard antibiotics and honey, lemon and honey/lemon juice mixture.

Plate II and III below shows the susceptibility test plates of *Streptococcus pneumoniae* with the largest zone of inhibition observed with honey/lemon juice mixture, undiluted lemon juice, Ceftriaxone and lowest zone shown by Azithromycin, Amoxicillin-Clavulanic acid and 50% v/v concentration of honey.

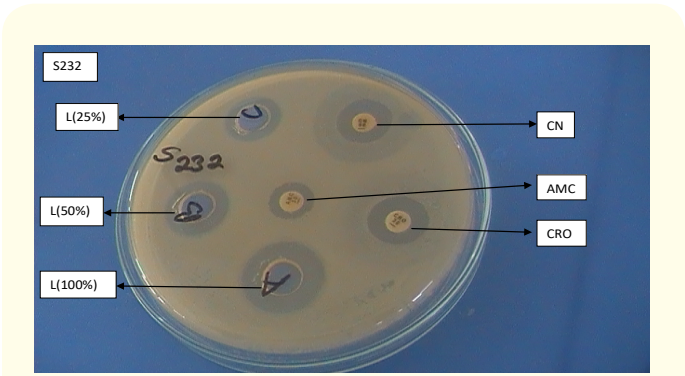


Plate II: Susceptibility Test plate of *Streptococcus pneumoniae*_1.
Key: L: Lemon Juice; CN: Gentamicin; AMC: Amoxicillin-Clavulanic Acid; CRO: Ceftriaxone.

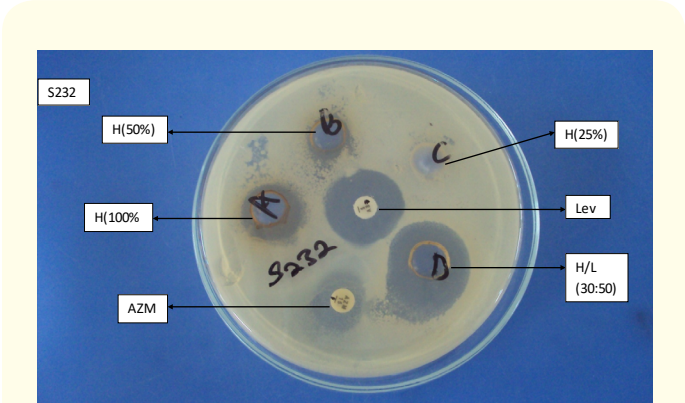


Plate III: Susceptibility Test plate of *Streptococcus pneumoniae*_2.
Key: H: Honey; Lev: Levofloxacin; AZM: Azithromycin; H/L: Honey and Lemon juice.

Discussion

All the tested bacterial isolates were completely susceptible to the crude concentrations of Honey, Lemon juice and the Honey/ Lemon juice mixture. This activity is tight to the acidic nature of the honey and lemon juice, beside other antimicrobial properties they were known to possess. They were also moderately susceptible to Honey at 50% v/v concentration, Lemon juice at 25% v/v concentration; but showed resistance to Honey at 25% v/v concentration. This is in agreement to works done by Ifra and Ahmad [29] and Kawaii., *et al.* [6] who reported that the stock solution of the honey samples inhibited the growth of all the bacterial isolates, but when the dilutions were made the efficacy reduced.

A better zone of inhibition was obtained with the honey/lemon juice mixture, crude concentrations of the honey and that of the lemon juice compared to the reduced concentrations. This is in close proximity to the work of Hemal., *et al.* [30], who reported that no significant result was found with 5%, 10%, 20% and 40% concentrations of honey solution. Zone of inhibition was observed with 60% concentration of honey solution, having 10.0 mm mean zone of inhibition. However, this is in contrast to a study by Faezeh., *et al.* [31], who observed that concentrations below 37.5 ppm were more efficient as antibacterials.

The minimum inhibitory concentrations which ranges from 20.0 - 75.0 (100% Honey), 22.5 - 47.5 (100% Lemon) and 17.5 - 25.0 (Honey/Lemon juice mixture) is in contrast to that of Faezeh., *et al.* [31], who reported a minimum inhibitory concentrations for honey against *Streptococcus mutans*, *Lactobacillus casei*, *L. rhamnosus* and *L. plantarum* at 75, 75, 100 and 100 ppm, respectively.

The rate of kill provides more accurate description of antimicrobial activity of antimicrobial agents than does the MIC [32]. The rate of kill of *Streptococcus pneumoniae* on exposure to the test agents showed that Honey/Lemon juice mixture and Lemon juice effected a better killing (evidenced by the sharp decrease in the bacterial cell populations and complete killing effect in less than 24 hours) than the Honey, which gave its complete killing at 24 hours. This is due to the highly acidic pH of the honey and lemon juice mixture. Kwakman., *et al.* [33] also reported in their work that antibiotic susceptible and resistant isolates of *S. aureus*, *S. epidermidis*, *Enterococcus faecium*, *E. coli*, *P. aeruginosa*, *E. cloacae* and *Klebsiella oxytoca* were killed within 24h by 10 - 40% (v/v) honey.

Generally, inadequate antimicrobial treatment defined as ineffective treatment due to failure to complete prescribed dosage or prescription without carrying out the susceptibility testing, use of left-over drugs and self-medication by buying drugs from the illegal conduits are important factors in emergence of antibiotic resistant bacteria [34]. This has motivated many to traditionally use the honey and lemon which can be found in their environment and has been found effective.

Therefore, honey and lemon juice mixture have better antibacterial activity than the honey or lemon juice when used alone.

Conclusion

Honey and lemon juice generally possess antibacterial activity against *Streptococcus pneumoniae* and *Streptococcus pyogenes* as obtained in this research. Honey and Lemon juice had more inhibitory effect against the tested bacterial isolates than the commonly used antibiotics especially Azithromycin and Amoxicillin-Clavulanic acid. Honey and Lemon juice can therefore be used as an alternative medicine in the treatment of respiratory tract infections.

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